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(54) Title: INHIBITORS OF CALCIUM INFLUX FACTOR SYNTHESIS AND/OR ACTION AND USES THEREOF

(57) Abstract

The present invention is directed to inhibitors of Calcium Influx Factor (CIF) synthesis and/or action and their potential applications as immunosuppressants, Th1/Th2 effectors, or therapeutic agents for cardiac hypertrophy. Glucosamine and metabolites are examples of such inhibitors.

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INHIBITORS OF CALCIUM INFLUX FACTOR SYNTHESIS AND/OR ACTION AND USES THEREOF

BACKGROUND OF THE INVENTION

Cross-reference to Related Application

This patent application claims benefit of provisional patent application U.S. Serial number 60/123,579, filed March 10, 1999.

Field of the Invention

The present invention relates generally to calcium physiology, the mechanism of action of immunosuppressants, Th1/Th2 effectors and cardiac hypertrophy. More specifically, the present invention relates to inhibitors of calcium influx factor (CIF) synthesis and/or action and uses thereof.

Description of the Related Art

The elevation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) that occurs when certain tyrosine kinase or G protein-linked receptors are engaged is usually initiated by activation of a phospholipase C and the formation of the second messenger inositol (1,4,5)-trisphosphate (IP_3) (Cardenas and Heitman, 1995). The interaction

of IP_3 with its receptor results in the release of stored Ca^{2+} from the endoplasmic reticulum (ER) lumen (Figure 1). This depletion of endoplasmic reticulum Ca^{2+} opens plasma membrane Ca^{2+} channels and leads to an influx of extracellular Ca^{2+} into the cell by a process referred to as store-operated or capacitative calcium entry (CCE).

Capacitative calcium entry is important to Ca^{2+}-mediated signal transduction because it strengthens short-term Ca^{2+} signals and provides cytoplasmic Ca^{2+} necessary for refilling depleted stores. In addition, in situations where long-lasting or repetitive ligand-receptor interactions result in continuous production of IP_3, capacitative calcium entry provides for the sustained elevation of $[Ca^{2+}]_i$ that activates specific transcriptional events not seen in response to transient stimuli. Primary among these is the transcription of genes regulated by members of the nuclear factor of activated T cells (NF-AT) protein family. Members of this family induce genes encoding a variety of cytokines including interleukin (IL)-2, IL-4, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and cell-surface receptors including CD40 and FasL.

The expression of cytokines induced by NF-AT family members is linked to the elevation of $[Ca^{2+}]_i$ through activation of the phosphatase calcineurin. When $[Ca^{2+}]_i$ rises, Ca^{2+}/calmodulin activated calcineurin dephosphorylates cytosolic substrates including NF-AT family members. These dephosphorylated transcription factors are then imported into the nucleus where they bind specific DNA targets and induce gene transcription. Dolmetsch et al. (1997) have shown that the magnitude and duration of Ca^{2+} signals determine which transcription factors are

translocated to the nucleus. The translocation of JNK and a subunit of NF-κB will occur in response to a brief spike in $[Ca^{2+}]_i$; in contrast, high sustained $[Ca^{2+}]_i$ is required to maintain NF-AT in the nucleus.

Metabolites within the hexosamine biosynthetic pathway (Figure 2) are increased when cells are exposed to a hyperglycemic environment. This pathway contributes to the development of insulin resistance (Marshall et al., 1991; Robinson et al., 1995) and has also been implicated as the nutrient-sensing pathway responsible for the regulation of leptin mRNA expression (Wang et al., 1998). Besides elevated blood glucose levels, an alternate means of stimulating this pathway is to provide cells or animals with exogenous glucosamine. Although not a normal serum constituent, glucosamine infusion of animals leads to serum levels sufficient to result in both insulin resistance (Rossetti et al., 1995) and an increase in leptin mRNA levels (Wang et al., 1998). In addition, glucosamine sulfate as a treatment for osteoarthritis has recently received widespread attention in both the scientific (Barclay et al., 1998; da Camara and Dowless, 1998) and lay press (Theodosakis et al., 1997). One explanation for its purported medicinal benefit is that increased metabolic flux from glucosamine to UDP-N-acetylglucosamine (UDP-GlcNAc) and other carbohydrate precursors of extracellular matrix constituents enhances the synthesis of glycosaminoglycans and other glycoconjugates thought to be required for the repair and maintenance of cartilage. This explanation assumes that the normal glucose flux through the hexosamine biosynthetic pathway is insufficient to provide adequate levels of metabolic precursors required for tissue repair and that exogenously supplied

glucosamine might provide higher concentrations of the necessary substrates. However, the experimental data that support this premise are limited.

The role of inflammation in the development of osteoarthritis is still being defined, but evidence is rapidly accumulating that implicates inflammatory cells and pro-inflammatory cytokines in the progression of this disease (Elson et al., 1998; Smith et al., 1997; Wagner et al., 1997; Westacott and Sharif, 1996).

Autoimmune diseases result from the destruction of host cells by immune responses directed against self-antigens. There are at least two checkpoints that normally keep autoimmunity within appropriate bounds. One occurs within the thymus, and is characterized by the activation-induced apoptosis of cells with T cell receptors (TCRs) that recognize appropriately presented self-peptides (Sprent, et al. 1988; Laufer, et al. 1999). The commitment to apoptosis appears to require a robust and prolonged response mediated by the engaged TCR (Iezzi, et al. 1998) and elevated $[Ca^{2+}]_i$ (Krebs 1998).

The second level of control occurs in the periphery. Some T cells specific for autoantigens escape thymic apoptosis but remain restrained in a self-tolerant state by immunoregulatory cytokine-dependent networks (Rabinovitch and Suarez-Pinzon 1998). When these networks fail, it is thought that autoreactive T cells expand and set in motion an immune/inflammatory spiral leading to cell destruction. Current thinking suggests that this pathogenic response occurs when the Th1 subset of T helper cells becomes dominant over the protective immune response mediated by Th2 cells. This balance is dependent upon NF-AT-dependent

cytokines. A host of studies demonstrate a shift to Th1 dominance in animal and human Type 1 diabetes, for instance (Rabinovitch and Suarez-Pinzon, 1998).

Cardiac hypertrophy is an adaptive response of the heart to virtually all forms of cardiac disease. Initially this compensatory pathway increases cardiac output. However, sustained hypertrophy can lead to reduced myocardial function, heart failure and death, and is characterized by myocyte enlargement and induction of fetal cardiac genes. A variety of factors that induce hypertrophy in cardiomyocytes are accompanied by elevations in $[Ca^{2+}]_i$. Recently, a link between elevated $[Ca^{2+}]_i$ and gene expression was established in cardiomyocytes with the discovery of a signaling pathway acting through Ca^{2+}/calcineurin and an NF-AT transcription complex (Molkentin, Lu et al. 1998; Sussman, Lim et al. 1998)

It has been also shown that transgenic expression of a constitutively active NFAT3 protein in the mouse heart produced hypertrophy (Molkentin, et al. 1998). In addition, a series of studies emerged demonstrating the ability of cyclosporine A (CsA) and FK506, inhibitors of calcineurin, were able to attenuate hypertrophy in cultured rat cardiomyocytes and various animal models (Molkentin, et al. 1998; Sussman, et al. 1998). However, the doses of FK506 required for prevention of cardiac hypertrophy are much higher than those required for immunosuppresson and produce kidney damage and other serious complications.

The prior art is deficient in calcium influx factor (CIF) inhibitors as immunosuppressants or immunomodulators, or as treatments or preventatives of cardiac hypertrophy. Further, the

prior art is deficient in methods of inhibiting capacitative calcium entry by CIF inhibitors, thereby inhibiting immune or hypertrophic responses. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

Calcium influx factor (CIF) is a small diffusible compound that is necessary for the ongoing activation of capacitative calcium influx. The present invention demonstrates that inhibitors of the synthesis and/or action of CIF inhibit capacitative calcium entry, and thereby inhibit immune responses and modulate cytokine production, therefore, alter immune function. This includes many of the processes that are currently inhibited by the immunosuppressive drug cyclosporin A. The present invention provides a new class of potential immunosuppressants utilizing a mechanism completely distinct from those in prior art. Such potential immunosuppressants are important to transplant rejection, autoimmune diseases such as arthritis and asthma, and in treating or preventing cardiac hypertrophy. Interfering with CIF's synthesis and/or action by CIF inhibitors should provide a novel approach to the treatment of these conditions.

The present invention also supports an alternative explanation for the action of glucosamine in the treatment of osteoarthritis and that glucosamine may be relevant to the importance of the hexosamine biosynthesis pathway to some complications of diabetes.

The present invention demonstrates that glucosamine inhibits capacitative calcium entry in calcineurin-containing cells. As a consequence of this inhibition, the predicted downstream sequelae, inhibition of NF-AT nuclear translocation and decreased synthesis of an NF-AT-mediated cytokine, were observed. These observations lead to the proposal that glucosamine, *via* its metabolite glucosamine-6-phosphate, acts as an immunomodulator by blocking store-operated Ca^{2+} channels and thereby disrupting Ca^{2+}-mediated cytokine expression in the broad array of cells (Rao et al., 1997) in which NF-AT family members influence gene transcription.

Additionally, since CIF would appear to play a critical role in determining the strength of response to T cell receptor (TCR) activation and influencing cytokine production, inhibitors of CIF synthesis or action will be effective in modulating Th1/Th2 balance and in regulating T cell apoptosis. These agents will therefore have value in not only immunosuppression but also in more general regulation of the immune system, especially with regard to autoimmune diseases.

The present invention demonstrates that CIF is active in excitable cells, in particular cardiac myocytes. In addition, it was observed that increased CIF activity is present in extracts prepared from dogs with hypertrophied cardiac tissue compared to controls. This indicates that inhibitors of CIF action or synthesis could be considered as novel therapeutic agents with respect to cardiac hypertrophy as well as immunosuppression or immunomodulation, due in part to their ability to inhibit NF-AT-mediated signaling.

In one embodiment of the present invention, there is provided a pharmaceutical compound which inhibits calcium influx factor (CIF) synthesis and/or action. CIF is required for capacitative calcium entry (CCE) and the downstream effects attributable to CCE, therefore, calcium influx factor inhibitors inhibit CCE. Representative examples of calcium influx factor inhibitors are glucosamine, metabolites of glucosamine, peptides that binds calcium-coordinating motifs and compounds that inhibits the activity of molecules responsible for opening calcium channel.

In another embodiment of the present invention, there is provided a method of suppressing immune responses in an individual in need of such treatment by administering the claimed pharmaceutical compound to the individual.

In still another embodiment of the present invention, there is provided a method of modulating Th1/Th2 balance or altering T cell apoptosis by administering the claimed pharmaceutical compound to an individual.

In still another embodiment of the present invention, there is provided a method of treating an individual having an impaired immune response condition by increasing intracellular free calcium concentration ($[Ca^{2+}]_i$) in selected cells within the individual, wherein the cells are associated with the impaired immune condition.

In still yet another embodiment of the present invention, there is provided a method of treating an individual having an impaired immune response condition by blocking hexosamine biosynthesis pathway in the individual, thereby

inhibiting synthesis and accumulation of glucosamine and/or its metabolites.

In yet another embodiment of the present invention, there is provided a method of treating an individual having cardiac hypertrophy or preventing an individual from developing cardiac hypertrophy by administering the claimed pharmaceutical compound to the individual.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows calcium signaling in immune cells.

Figure 2 shows the hexosamine biosynthesis pathway. Metabolism into the hexosamine biosynthesis pathway originating from either extracellular glucose (Glc) or glucosamine

($GlcNH_2$) is shown. GFAT is the rate limiting enzyme glutamine:fructose-6-phosphate amidotransferase.

Figure 3 shows that glucosamine inhibits capacitative calcium entry in a concentration-dependent manner and is specific. **Figure 3A** shows Jurkat T cells loaded with Fura-2 were incubated for 7 minutes in HBSS supplemented with varying concentrations of glucosamine. The $[Ca^{2+}]_i$ response to 100 nM thapsigargin (Tg) (arrow) in the presence of 1.25 mM extracellular Ca^{2+} is shown. **Figure 3B** shows Ca^{2+} responses of untreated Jurkat T cells or those pretreated with 25 mM glucosamine to 100 nM Tg (arrow) in the absence and presence of 2.5 mM extracellular Ca^{2+} (arrowhead). **Figure 3C** shows capacitative calcium entry by BHK-21 cells pretreated with varying concentrations of glucosamine for 2 minutes and then stimulated with 100 nM Tg (arrow). Experiments shown in each case are representative of three independent trials. **Figure 3D** shows Jurkat T cells loaded with Fura-2 were incubated for 7 minutes in HBSS supplemented with 25 mM glucosamine, galactosamine, ethanolamine, GlcNAc, or 2-deoxyglucose as indicated. Capacitative calcium entry was initiated with 100 nM Tg (arrow).

Figure 4 shows that glucosamine does not decrease total cellular ATP and is not cytotoxic. **Figure 4A** shows that perchloric acid extracts of Jurkat T cells ($2x10^7$ cells per assay) cultured RPMI-1640 in the absence (control) or presence (glucosamine) of 10 mM glucosamine for 30 minutes were analyzed by anion-exchange HPLC using a linear gradient of 15 mM H_3PO_4, pH 3.8 to 1 M H_3PO_4, pH 4.5 over 50 minutes at a flow rate of 1 ml/min. Retention times of metabolites were detected by monitoring UV absorbance at 260 nm and comparing to

retention times of standards as indicated. **Figure 4B** shows an autoradiograph of Jurkat T cells extracts prepared from cultures that were treated with varying concentrations (mM) of glucosamine as shown for eight hours, and metabolically labeled with Tran[^{35}S]label for the last hour. The eletrophoretic mobility of standards is shown (arrowheads) and the position of two newly appearing bands with mobilities consistent with GRP78 and GRP94 are indicated by arrows. **Figure 4C** shows Jurkat T cells loaded with Fura-2 were incubated for 7 minutes in HBSS in the presence (Glucosamine) or absence (Control) of 25 mM glucosamine. After a 7 minute incubation, glucosamine was removed from some cells by centrifugation, and the cells allowed to recover in HBSS for 10 minutes (Recovery). The $[Ca^{2+}]_i$ response to 100 nM Tg (arrow) is shown. The experiment shown is typical of three replicates.

Figure 5 shows stimulus-induced nuclear translocation of GFP-NF-AT4 is inhibited by glucosamine. BHK-21 cells were transfected with a plasmid encoding GFP-NF-AT4. GFP-NF-AT4 translocated from the cytosol (**Figure 5A**) to the nucleus (**Figure 5B**) following a 10 minute treatment with Tg. Nuclear translocation was reversed within 15 minutes of treatment with 25 mM glucosamine (**Figure 5C**). When cells were pretreated with glucosamine, nuclear translocation of GFP-NF-AT4 in response to thapsigargin was completely abrogated (**Figures 5D/E**). However, GFP-NF-AT4 nuclear import was observed in these cells when they were subsequently treated with A23187, demonstrating that the translocation machinery was intact (**Figure 5F**). In all cases, nuclear translocation could be reversed by 1 mM EGTA.

Figure 6 shows that NF-AT-mediated cytokine expression is inhibited by glucosamine and is reversed when [Ca^{2+}] is elevated. **Figure 6A** shows Jurkat T cells were treated with varying concentrations (mM) of glucosamine or mannitol as indicated and stimulated with phorbol myristate acetate (PMA)/phytohemagglutinin (PHA). The amount of IL-2 secreted into the media after 8 hours was determined as described below. Results are expressed as a percentage of IL-2 secreted in the absence of glucosamine and are the mean of three determinations ± standard errors of the mean. **Figure 6B** shows IL-2 secreted into the media by Jurkat T cells was measured as described below. Cells were cultured in either 0, 2.5, or 5 mM glucosamine as indicated in either the absence (solid bars) or presence of A23187 (hatched bars) or 10 mM extracellular Ca^{2+} (open bars). Results are expressed as the average of triplicate measurements ± standard errors of the mean.

Figure 7 shows that glucosamine-6-phosphate inhibits the trans-plasma membrane current referred to as I_{CRAC}. **Figure 7A** shows a time course of activation of an RBL-2 cell in the whole-cell configuration. The slowly activating current (a, c) is permeable to Na^+ in the absence of all divalent cations (b), and is closed in the absence of Ca^{2+} when Mg^+ is present (d). **Figure 7B** shows current-voltage relationships recorded at the time points indicated in Figure 7A. **Figure 7C** shows whole-cell patch clamp recording of an RBL-2 cell prior to (a) and following the addition of 10 mM glucosamine (b) to the extracellular solution when indicated. **Figure 7D** shows current-voltage relationships recorded at the time points indicated by a and b in Figure 7C. **Figure 7E** shows whole-cell patch clamp recording of an RBL-2

cell with a pipette containing hexokinase and ATP. 10 mM glucosamine (b) was added to the extracellular solution when indicated and caused a decrease in I_{CRAC} relative to the control (a). **Figure 7F** shows current-voltage relationships recorded at the time points indicated by a and b in Figure 7E. **Figure 7G** shows time course of activation of I_{CRAC} in an RBL-2 by 50 μM IP_3 in the absence or presence of 10 mM glucosamine-6-phosphate present in the pipette. **Figure 7H** shows the magnitude of I_{CRAC} 240 seconds after the whole-cell configuration was achieved (a) and in the presence of 10 mM (b) glucosamine, (c) glucosamine-6-phosphate, (d) GlcNAc-6-P, (e) GlcNAc-1-P, or (f) UDP-GlcNAc present in the pipette. Magnitude of I_{CRAC} 240 seconds after the whole cell configuration was achieved in the presence of (g) 0.1 mM, (h) 1 mM, or (i) 10 mM glucosamine-6-phosphate in the pipette or (j) 10 mM glucosamine-6-phosphate in the extracellular bath. Results are expressed as means of at least 4 experiments in each case ± standard errors of the mean.

Figure 8 shows a comparison of the time courses of I_{CRAC} activation by IP_3, and cellular extracts that contain or do not contain CIF. The time courses of the activation of I_{CRAC} by dialysis of the cytosol of a Jurkat T cell with IP_3, a cellular extract that did not contain CIF, and a cellular extract containing CIF were quantified. The times to achieve an I_{CRAC} of 7.5 pA in Jurkat T cells by the cellular extract that did not contain CIF and 10 μM IP_3 were 120 and 56 seconds, respectively. In contrast, the activation of I_{CRAC} induced by the CIF-containing extract was much more rapid, reaching 7.5 pA by 17 seconds. The nearly immediate activation of I_{CRAC} by a CIF-containing is consistent with a model in which CIF is active in the signaling cascade at a step downstream of IP_3.

Figure 9 shows that peripheral blood mononuclear cells (PBMCs) from diabetic individuals secrete lower levels of NF-AT mediated cytokines following activation than do those from controls. IL-2 (**Figure 9A**), IL-4 (**Figure 9B**), IL-10 (**Figure 9C**), granulocyte-macrophage colony-stimulating factor (GM-CSF) (**Figure 9D**) and INF-γ (**Figure 9E**) were tested.

Figure 10 shows that capacitative calcium entry (CCE) elicited by PHA is decreased in peripheral blood mononuclear cells prepared from diabetic individuals compared to euglycemic controls.

Figure 11 shows that CCE elicited by thapsigargin (Tg) is decreased in peripheral blood mononuclear cells prepared from diabetic individuals compared to euglycemic controls. This shows that the defect in diabetes is at least in part distal to release of Ca^{2+} from intracellular stores.

Figure 12 shows that CCE elicited by PHA is decreased in peripheral blood mononuclear cells prepared from diabetic individuals compared to euglycemic controls. The intracellular release of Ca^{2+} was similar in response to PHA (indicated by arrow) in the absence of extracellular Ca^{2+}. When calcium was added back (indicated by arrow), the capacitative influx was decreased in cells prepared from the diabetic group compared to those from controls.

Figure 13 shows that T cells from diabetic patients have a markedly reduced I_{CRAC}.

Figure 14A shows that introduction of an eight amino acid peptide (CALP1, SEQ ID No. 1) that binds to a conserved domain (EF hand) responsible for the coordination of calcium is able to directly inactivate a non-selective cation channel. This

channel is present in Jurkat T cells, is activated by gp120, and contributes to the inward Ca^{2+} current that characterizes capacitative calcium entry. The current voltage relationship of the current activated by gp120 in the presence and absence of 40 μM CALP1 is shown. **Figure 14B** shows that I_{CRAC} activated by whole cell passive dialysis of Jurkat T cells is blocked by CALP1. The current voltage relationships of 40 μM I_{CRAC} before and I min. following the addition of CALP1 are shown. In all cases, the leak currents have been subtracted.

Figure 15 shows (^{3}H)thymidine incorporation and cytokine production in response to antigen stimulation by lymphocytes cultured in the presence of glucosamine. It shows that the presence of 2 mM glucosamine caused no significant change in (^{3}H)thymidine incorporation (**Figure 15A**) but caused a significant shift toward a Th1 bias, as assessed by the increased IL-2 and decreased IL-10 production at 72 hr (**Figure 15B**).

Figure 16 shows IL-2 production in response to antigen stimulation by lymphocytes cultured in the presence of varying concentrations of glucosamine. It shows that in cultures from all five rats and at all glucosamine concentrations examined, the production of IL-2 at 18 hours was about 50% above control levels.

Figure 17 shows that CIF is present in extracts from hypertrophied heart.

DETAILED DESCRIPTION OF THE INVENTION

The present invention examines the hypothesis that elevated hexosamine biosynthesis due to hyperglycemia or

dietary glucosamine impairs NF-AT-mediated immune responses by inhibiting stimulus-induced capacitative calcium entry. Glucosamine serves as a prototype for compounds that inhibit the synthesis and/or action of calcium influx factor. Glucosamine, in a concentration-dependent manner, rapidly inhibited capacitative calcium entry elicited by lectins or thapsigargin in Fura-2-loaded Jurkat T lymphocytes and BHK-21 cells. Glucosamine treatment inhibited nuclear translocation of a transfected green fluorescent protein-NF-AT4 chimera and IL-2 production. These effects were attributed to impaired calcium responses as they were reversed by a calcium ionophore. Levels of cytosolic glucosamine-6-phosphate increased following brief glucosamine treatments, but there were no changes in ATP and UDP-N-acetylglucosamine. Whole-cell patch clamp experiments determined that the initial intracellular glucosamine metabolite, glucosamine-6-phosphate, inhibited I_{CRAC}, the trans-plasma membrane current initiated by calcium store depletion.

The present invention also determined whether the impaired immune responses that characterize diabetes is related to impaired Ca^{2+} signaling and, in turn, if this impairment is due to enhanced levels of intermediates in the hexosamine biosynthetic pathway. The data imply that the hexosamine biosynthetic pathway is responsible for many of the defects in immune function seen in diabetes and further appears to be particularly relevant to the widespread use of glucosamine as a therapy for osteoarthritis. As an objective of the present study, it was also investigated whether exogenous glucosamine used as an alternative remedy in the treatment of osteoarthritis in humans is sufficient to give rise to impaired $[Ca^{2+}]_i$ signaling in immune cells.

Furthermore, the present invention demonstrates that glucosamine is able to prevent or slow the progression of autoimmune diseases characterized by inflammation.

Cyclosporin A is the primary immunosuppressive drug currently used for transplant rejection. However, there are many complications that are part of the presently available immunosuppressive protocols. Compounds that are active in inhibiting the synthesis and/or action of CIF disclosed in the present invention appear to have less toxicity than cyclosporin A and to work synergistically with cyclosporin A. Therefore, it may be possible to use cyclosporin A at a lower concentration and thus reduce its toxicity, or to utilize the compounds geared toward the inhibition of CIF's synthesis and/or action and eliminate the complications associated with current immunosuppressive protocols.

The following abbreviations may be used herein:

$[Ca^{2+}]_i$	intracellular free calcium concentration
CCE	capacitative calcium entry
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethyleneglycol tetra acetic acid
ER	endoplasmic reticulum
GFP-NF-AT4	green fluorescent protein-NF-AT4
GlcNAc	N-acetylglucosamine
HBSS	Hank's balanced salt solution
HPLC	high performance liquid chromatography
I_{CRAC}	calcium release activated calcium current
IL-2	interleukin-2
IP_3	inositol (1,4,5)-trisphosphate
MEM	minimum essential medium

NF-AT	nuclear factor of activated T cells
PBMCs	peripheral blood mononuclear cells
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
SDS-PAGE	sodium doedecyl sulfate-polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum

In one embodiment of the present invention, there is provided a pharmaceutical compound which inhibits calcium influx factor (CIF) synthesis and/or action. Calcium influx factor is required for capacitative calcium entry (CCE) and the downstream effects attributable to CCE, therefore, calcium influx factor inhibitors inhibits CCE. Representative examples of calcium influx factor inhibitors are glucosamine, metabolites of glucosamine, a peptide that binds calcium-coordinating motifs and a compound that inhibits the activity of molecules responsible for opening calcium channel. Preferably, the glucosamine metabolite is glucosamine-6-phosphate, and the peptide is CALP1 having the amino acid sequence shown in SEQ ID No. 1. More preferably, such inhibitors function in a concentration-dependent manner.

In another embodiment of the present invention, there is provided a method of suppressing immune responses in an individual in need of such treatment by administering the claimed pharmaceutical compound to the individual. Preferably, the individual suffers from transplant rejection, autoimmune diseases like multiple sclerosis, diabetes, arthritis and asthma, or other inflammation associated diseases like osteoarthritis, or a condition in which immunosuppression is therapeutic. In such method, the

pharmaceutical compound inhibits NF-AT-mediated gene expression, such as IL-2, IL-4, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Fas ligand and CD-40 expression and other calcium-sensitive transcription pathways.

In still another embodiment of the present invention, there is provided a method of modulating Th1/Th2 balance or altering T cell apoptosis by administering the claimed pharmaceutical compound to an individual. In such method, the pharmaceutical compound inhibits CCE and its downstream consequences.

In still another embodiment of the present invention, there is provided a method of treating an individual having an impaired immune response condition by increasing intracellular free calcium concentration ($[Ca^{2+}]_i$) in selected cells within the individual, wherein the cells are associated with the impaired immune condition.

In still yet another embodiment of the present invention, there is provided a method of treating an individual having an impaired immune response condition by blocking hexosamine biosynthesis pathway in the individual, thereby inhibiting synthesis and accumulation of glucosamine and/or its metabolites.

In a preferred embodiment, the impaired immune response condition is caused by hyperglycemia or dietary glucosamine. More preferably, hyperglycemia is associated with the condition selected from the group consisting of diabetes, traumatic injuries (particularly burns), steroid treatment, total parenteral nutrition treatment and end-stage renal failure treatment which utilizes excessive glucose in the dialysis solution.

In yet another embodiment of the present invention, there is provided a method of treating an individual having cardiac hypertrophy or preventing an individual from developing cardiac hypertrophy by administering the claimed pharmaceutical compound to the individual. In such method, the pharmaceutical compound inhibits CCE and its downstream consequences.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion:

EXAMPLE 1

Materials

Glucosamine was purchased from Fluka Chemical Co. (Milwaukee, WI), Fura-2AM was purchased from Molecular Bioprobes (Eugene, Oregon), fetal calf serum was from InVitrogen, and lipofectin was from Gibco/BRL Life Technologies (Grand Island, NY). All other chemicals were of the highest available grade and were purchased from Sigma Chemical Corporation (St. Louis, MO). BHK-21 and Jurkat T cell lines were purchased from the American Type Culture Collection (Manassas, VA). The plasmid encoding GFP-NF-AT4 was the gift of Dr. Frank McKeon, Harvard University, Boston, MA.

EXAMPLE 2

Cell Culture

BHK-21 cells were cultured 75 mm^2 flasks in minimal essential medium (MEM) supplemented with 2 mM glutamine, sodium bicarbonate, 10% fetal calf serum and penicillin-streptomycin referred to as complete BHK-21 medium. The cells

were passaged using a brief treatment with trypsin-EDTA. Jurkat T lymphocytes were cultured in 75 mm^2 flasks in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin referred to as complete Jurkat T medium. All cells were cultured in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Cells used for microscopic analysis were cultured on LabTek chambered coverglasses (Nunc, Inc.).

EXAMPLE 3

Measurements of $[Ca^{2+}]_i$

BHK-21 cells were released from the tissue culture flasks by treatment with trypsin-EDTA and resuspended into serum-free MEM (2 x 10^6 cells/ml). The cells were washed by centrifugation at 300 x g for 3 min, and incubated in serum-free MEM containing 2 µM Fura-2AM for 30 minutes, 37°C. Excess Fura-2AM was removed by washing the cells once into MEM containing 1% bovine serum albumen, once into HBSS, followed by a final wash into HBSS containing 2.5 mM probenecid to prevent the leak of Fura-2 from the cells (Sung and Silverstein, 1985). The cells were incubated on ice for 30 minutes prior to use. Individual aliquots of Jurkat T cells were concentrated to 4 x 10^6 cells/ml by centrifugation, resuspended in RPMI-1640 supplemented with 2% fetal bovine serum containing 2 µM Fura-2AM for 30 minutes, 37°C. Excess Fura-2AM was removed by washing the cells once into HBSS and resuspending the cells in HBSS to a final concentration of 2x10^6 cells/ml.

$[Ca^{2+}]_i$ was measured using a spectrofluorometer (PTI System QM-1) with alternating excitation at 340 and 380 nm, and fluorescence emission at 510 nm. After a baseline level of $[Ca^{2+}]_i$

was established, the cells were treated with either 10 μg/ml PHA or 100 nM thapsigargin. When a stable plateau of $[Ca^{2+}]_i$ in response to either agent was achieved, the cells were permeabilized with digitonin. EGTA was then added to a final concentration of 5 mM. The digitonin and EGTA measurements were used to calibrate $[Ca^{2+}]_i$ versus Fura-2 fluorescence in each sample using a calibration equation (Grynkiewicz et al., 1985). Some experiments employed a Ca^{2+} removal/Ca^{2+} addback protocol described (Rivera et al., 1995).

EXAMPLE 4

Nuclear Translocation of GFP-NF-AT4 in BHK-21 Cells

The plasmid encoding the chimeric protein GFP-NF-AT4 (Shibasaki et al., 1996) was introduced into BHK-21 cells using lipid transfection. Briefly, complete BHK-21 medium was removed, and the cells were incubated in Optimem I containing 20 μg/ml DNA using either a 1:1 mixture of the cationic lipids DMRIE:DOPE (10 μg/ml) or lipofectin (10 μg/ml) for 16 hours. Following the transfection period, the medium was removed and replaced with complete medium. Forty-eight hours after transfection, the medium was removed, and replaced with HBSS. The expression of GFP-NF-AT4 was visualized on a heated stage of an Olympus 1x70 inverted microscope illuminated by an Optiquip hybrid mercury/xenon lamp. Nuclear translocation of GFP-NF-AT4 was observed using an FITC filter and recorded using a Photometrics Sensys cooled CCD camera (12 bit) linked to a Power Mac 9500 (132 mHz, 96 MB RAM) loaded with IPLab spectrum software.

EXAMPLE 5

Measurement of IL-2 Secreted by Jurkat T cells

$2x10^6$ Jurkat T cells were cultured in RPMI-1640 supplemented with either 0, 2.5, 5, 10, 15, 25 mM glucosamine or 25 mM mannitol as a control. NaCl was used to adjust the osmolarity of the sugars to 25 mM. After a 15 min preincubation in glucosamine or mannosamine-containing medium, cultures were either stimulated with 16 nM phorbol myristate acetate (PMA) and 20 μg/ml phytohemagglutinin (PHA) or left untreated and incubated at 37°C. Eight hours following the stimulation, the media was collected a brief centrifugation, the supernatant was filtered using a 0.2 micron filter, and the amount of IL-2 secreted was measured using a commercial kit (Quantikine, Human IL-2 Immunoassay, R&D Systems). In some experiments, intracellular Ca^{2+} was elevated by supplementation of either 100 mM $CaCl_2$ or 100 nM A23187 in the extracellular medium.

EXAMPLE 6

Metabolic Labeling of Jurkat T Lymphocytes

Jurkat cells were treated with glucosamine and stimulated with PMA/PHA as described above. After seven hours of stimulation, 100 μCi/ml Tran[^{35}S]-label was added to the medium for an additional hour. Cells were collected by centrifugation, washed once with PBS. Aliquots of $2x10^5$ cells were lysed in boiling 4X SDS-Page sample buffer. Equal aliquots from each sample were loaded onto 10% discontinuous SDS-polyacrylamide gels and electrophoresed. Proteins were electrically transferred to Immobilon-P and the pattern of

macromolecular incorporation of the label was determined by autoradiography and compared to protein standards (Gibco/BRL).

EXAMPLE 7

Whole-Cell Patch Clamp Recording

Cell preparation: RBL-2 cells were maintained as described (Parekh and Penner, 1995). After two washes with Ringer's solutions, the cells were resuspended and allowed to settle onto glass coverslips for 10 min. The nonadherent cells were then removed by washing the coverslip twice with Ringer's solution. Coverslips were broken into small pieces, and these fragments were put onto the bottom of the recording chamber (volume = 30 µl).

Whole-cell patch clamp experiments: Conventional whole-cell recording was performed on a List EPC-7 patch clamp amplifier (LIST-Medical, Germany). Pipettes were pulled from KG12 capillaries (World Precision Instrument, Inc, Sarasota, Florida), Q-Dupe coated and fire polished to produce a tip resistance of 2-5 MΩ in Ringer's solution. The pipette solution (cytoplasmic-like solution) contained 110 mM CsGlutamate, 2.9 mM $MgCl_2$, 0.6 mM $CaCl_2$, 10 mM EGTA-Cs, 10 mM HEPES, pH 7.2 adjusted with CsOH. The bath solution contained 143 mM NaCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 4.5 mM KCl, 5 mM D-glucose, 10 mM HEPES, pH 7.3 adjusted with NaOH. Nominally Ca^{2+} free bath solution was made by replacing $CaCl_2$ with equal molar $MgCl_2$. The solution exchange in the chamber was achieved by mobile linear array puffer pipettes, and was completed in less than 200 msec. The fast and slow capacitative transients were canceled using the facility of EPC-7, and a tip junction potential of +10 mV was not

corrected. After the whole cell configuration was achived, the cells were held at -40 mV, and data were sampled at 5 kHz on a magnetic tape. For display, the signal was filtered at 1000 Hz by an eight-pole low-pass Bessel filter Model 900, Frequency device, USA). A 320 ms voltage ramp from -100 to +100 mV at frequency of 0.2 Hz was applied to the cell to obtain the current-voltage relationship. The data were analyzed by PCLAMP 7.0 (Axon Co., CA). The currents measured at -80 mV were displayed in Figure 7.

EXAMPLE 8

Measurement of Intracellular Metabolites

Jurkat T cells were extracted with 0.1 M HCl at 4°C for 30 minutes and the extracts centrifuged for 30 minutes at 15,000 x g. The supernatants were subjected to anion-exchange HPLC chromatography over a Keystone Partisil 10 SAX column using linear salt and pH gradients from 5 to 750 mM ammonium dihydrogen phosphate and from pH2.8 to 3.7, respectively. This chromatography step has been used successfully to resolve nucleotide phosphates (Pogolotti and Santi, 1982).

EXAMPLE 9

Glucosamine Treatment Inhibits CCE

Whether varying concentrations of extracellular glucosamine would affect the capacitative calcium entry that is observed in Jurkat T cells when Ca^{2+} signaling is initiated with the lectin phytohemagglutin (PHA) was first examined. This lectin aggregates cell-surface receptors, initiates IP_3 signaling, and results in the influx of Ca^{2+} from the extracellular milieu (Putney,

1990). As shown in Fig. 3A, the $[Ca^{2+}]_i$ response to PHA was rapid and included a sustained elevation of $[Ca^{2+}]_i$ that persisted with time. When the cells were pretreated briefly (7 minutes) with varying concentrations of glucosamine, a dose-dependent attenuation of capacitative calcium entry initiated by PHA treatment was observed.

To determine if glucosamine was inhibiting capacitative calcium entry at a step prior to the release of Ca^{2+} from intracellular stores, capacitative calcium entry was initiated in a similar series of experiments but using the irreversible ER Ca^{2+}ATPase inhibitor thapsigargin (Tg) (Thastrup et al., 1990). A rapid and sustained increase in $[Ca^{2+}]_i$ was observed in control cells exposed to Tg (Figure 3B). However, when cells pretreated with varying concentrations of glucosamine were exposed to Tg, a rapid initial rise in $[Ca^{2+}]_i$ was observed that then decayed to a plateau level that was decreased from that of control cells. Glucosamine was effective in reducing capacitative calcium entry initiated by Tg over concentrations ranging from 1 to 25 mM in the presence of the 5 mM glucose in HBSS.

A Ca^{2+} removal/Ca^{2+} addback protocol was also utilized to examine separately the effects of glucosamine on the release of Ca^{2+} from intracellular stores and capacitative influx. Cells were treated with Tg in the nominal absence of extracellular Ca^{2+} so that the rise in $[Ca^{2+}]_i$ directly reflects the Ca^{2+} released from intracellular compartments. Once this initial response has decayed due to efflux of Ca^{2+} at the plasma membrane, Ca^{2+} is added to the extracellular bath. The peak $[Ca^{2+}]_i$ observed due to release of intracellular stores by Tg treatment was not substantially different in the glucosamine pretreated group (Figure 3C). When

Ca^{2+} was added to the extracellular buffer, a robust increase in intracellular Ca^{2+} was observed in the control cells. In contrast, only a small initial rise in $[Ca^{2+}]_i$ was observed in the glucosamine treated group, and the plateau value was greatly reduced. The decrease in capacitative calcium entry was not specific to cells derived from the T cell lineage, as a similar dose-dependent inhibition with glucosamine was seen after a 2 min treatment of the baby hamster kidney (BHK)-21 cell line (Figure 3C).

EXAMPLE 10

The Inhibitory Effect Of Glucosamine On CCE Is Specific, Not Due To General Toxicity, And Reversible

Other sugars and amino sugars were tested for their ability to inhibit capacitative calcium entry including ethanolamine, galactosamine, mannoheptulose, galactose, N-acetylglucosamine, mannitol, and 2-deoxyglucose. Of the compounds tested, only 2-deoxyglucose was strongly inhibitory (Figure 3D). The effect of 2-deoxyglucose on capacitative calcium entry has been previously reported and is attributable to a decrease in glycolytically generated ATP (Darbha and Marchase, 1996). Interestingly, some inhibition of the capacitative calcium entry plateau was observed in Jurkat T cells pretreated with galactosamine. Although the initial peak value was comparable to control cells, the subsequent $[Ca^{2+}]_i$ levels slowly dropped with time. This could be due to reduced kinetics for either the intracellular entry of galactosamine or enzymatic conversion to the active metabolite.

To determine whether the inhibitory effect of glucosamine on capacitative calcium entry was a reflection of a

general, deleterious effect on cell health, protein synthesis patterns and whether the glucosamine inhibition was reversible were examined. Whether, in the time scale required for the observed inhibition, glucosamine treatment led to a depletion in intracellular ATP levels was also examined. Figure 4A shows elution profiles of acid extracts resolved by anion-exchange high performance liquid chromatography (HPLC) that were prepared from control cells and cells treated with 10 mM glucosamine for 30 minutes. After this incubation period, no decreases in ATP in other trinucleotides were observed, although a time-dependent increase in UDP-GlcNAc was seen in extracts prepared from the glucosamine-treated cells compared to those from controls. After a three-hour incubation, a more significant increase in UDP-GlcNAc was observed, although there was no change in ATP/ADP. In order to examine other metabolites, cells were labeled with 10 mM [^{3}H]glucosamine during the incubation period. Radioactive peaks corresponding to the retention times observed for authentic glucosamine and glucosamine-6-phosphate were quantitated. Radioactivity that co-eluted with glucosamine-6-phosphate amounted to 0.03 $\pm$ 0.01 pmols/10^6 cells. This was approximately one fourth the amount of ATP in the extracts and suggests an overall intracellular concentration of approximately 0.2-0.5 mM for glucosamine-6-phosphate.

To determine if the inhibitory effect of glucosamine on capacitative calcium entry was reflecting a more general effect on cell viability, Jurkat T cells were stimulated with PMA/PHA and cultured in the presence of varying concentrations of glucosamine. The proteins synthesized during the last hour of the 8 hr treatment were metabolically labeled with [^{35}S]methionine and

compared by SDS-PAGE (Figure 4B). The patterns of radioactive proteins were nearly identical, demonstrating that at even the higher glucosamine concentrations the cells were metabolically active. However, the appearance of two new protein bands with apparent molecular sizes of 94 and 78 kDa was observed. The expression of these proteins increased with increasing concentrations of glucosamine. They have been tentatively identified as the ER stress proteins GRP94 and GRP78 which have been reported to be induced by glucosamine (Price et al., 1992) and when intracellular Ca^{2+} stores are depleted by a variety of agents including treatment with EDTA (Lin et al., 1993) and thapsigargin (Li et al., 1993; Price et al., 1992).

Lastly, using the Fura-2 assay, whether the glucosamine-inhibition of CCE was reversible when the glucosamine-containing media was replaced with standard buffer was examined. Nearly a complete recovery of capacitative calcium entry was restored following the replacement of glucosamine-containing media (Figure 4C). Taken together, these data demonstrate that glucosamine inhibition of capacitative calcium entry is specific, rapidly reversible, and not a result of either ATP depletion or a general cytotoxic effect.

EXAMPLE 11

GFP-NF-AT4 Nuclear Translocation And NF-AT Gene Transcription Is Inhibited By Glucosamine

The reduced $[Ca^{2+}]_i$ response observed in glucosamine-treated cells led to the prediction that downstream events dependent on a sustained elevation in $[Ca^{2+}]_i$ would be decreased or inhibited. As a first step in evaluating this prediction, the

translocation of NF-AT from cytosol to nucleus in response to stimulation in the presence or absence of glucosamine was examined. BHK-21 cells were transfected with a plasmid encoding a chimeric protein in which an enhanced green fluorescent protein (Cubitt et al., 1995) is linked to the amino terminus of NF-AT4. This construct has been previously reported to maintain the translocation properties of NF-AT family members, moving from the cytosol to the nucleus when intracellular calcium is elevated with the calcium ionophore A23187 (Shibasaki et al., 1996). The nuclear translocation of GFP-NF-AT4 in response to 100 nM Tg was rapid and complete within 6 minutes of treatment in greater than 80% of the cells examined that expressed the chimeric protein (Figures 5A and 5B). A subsequent stimulation with 25 mM glucosamine reversed this nuclear import (Figure 5C). Interestingly, nuclear localization could be restored by a further treatment with A23187 (Figure 5F), suggesting that the translocation machinery was intact and not directly inhibited by glucosamine. When cells were pretreated with glucosamine, nuclear translocation of GFP-NF-AT4 in response to Tg treatment was completely abrogated (Figures 5D and 5E). Again, GFP-NF-AT4 nuclear import was observed in these cells when they were subsequently treated with A23187 (Figure 5F). In all cases, nuclear translocation could be reversed by treatment with 1 mM EGTA.

The stimulus-induced production of an NF-AT mediated cytokine was measured in the presence or absence of glucosamine. In order to assess this, Jurkat T cells were stimulated with PMA/PHA and cultured in the presence of varying concentrations of glucosamine. Following an 8 hr

exposure, glucosamine effectively inhibited IL-2 secretion over a range of concentrations from 2.5 to 25 mM (Figure 6A). No IL-2 was detected in the media of unstimulated control cells.

EXAMPLE 12

The Inhibitory Effect Of Glucosamine On IL-2 Expression Is Reversed By Elevating $[Ca^{2+}]_i$

Raising $[Ca^{2+}]_i$ *via* a pathway independent of capacitative calcium entry or elevating extracellular Ca^{2+} overcame the inhibition of IL-2 secretion that occurs in the presence of glucosamine. Jurkat T cells were either left untreated or treated with 2.5 or 5 mM glucosamine for 15 min. The cells were then stimulated with PMA/PHA to induce IL-2 expression in the presence or absence of either high extracellular Ca^{2+} (10 mM) or normal Ca^{2+} and 100 nM of the calcium ionophore A23187. After 8 hours, the amount of IL-2 secreted into the medium was measured. As seen in Figure 6B, both means of raising $[Ca^{2+}]_i$ were sufficient to overcome the glucosamine-mediated inhibition of IL-2 synthesis.

EXAMPLE 13

An Intracellular Metabolite Of Glucosamine, Glucosamine-6-Phosphate, Inhibits I_{CRAC}

Whether the inhibitory effect of glucosamine on capacitative calcium entry as determined by imaging techniques could also be seen in whole-cell patch clamp recordings was examined. In order to activate capacitative calcium entry, RBL-2 cells were dialyzed with a pipette solution containing Ca^{2+} buffered to 20 mM. Under these conditions, a Ca^{2+} current

referred to as Ca^{2+} release activated Ca^{2+} current (I_{CRAC}) is activated in this cell (Figures 7A and 7B). The current was inwardly rectified with a reversal potential near +60 mV (Figure 7B).

When whole-cell patch clamp recordings were performed on RBL-2 cells with 10 mM glucosamine present in the extracellular solution, no inhibition of I_{CRAC} was seen (Figures 7C and 7D). This excludes the possibility that glucosamine was inhibiting I_{CRAC} and thus CCE by acting directly on the extracellular face of the responsible plasma membrane channels. It also suggests that cytosolic glucosamine is not the inhibitory metabolite. In the whole cell configuration, the cytosol is equilibrated with the large intra-pipette volume, thus diluting ATP and other soluble intracellular constituents. However, when hexokinase and ATP were included in the pipette solution, extracellular glucosamine partially inhibited I_{CRAC} (Figures 7E and 7F). This suggested that the conversion of glucosamine to glucosamine-6-phosphate by hexokinase was required in order to create the inhibitory metabolite. To assess this possibility directly, pre-formed glucosamine-6-phosphate was included in the pipette solution. The activation of I_{CRAC} by 50 μM IP_3 was inhibited when 10 mM glucosamine-6-phosphate was present in the pipette solution (Figure 7G). The magnitude of I_{CRAC} was inhibited in a dose-dependent manner by glucosamine-6-phosphate in the pipette but not by extracellular glucosamine-6-phosphate (Figure 7H). These data indicate that glucosamine-6-phosphate is the metabolite in the hexosamine pathway responsible for inhibiting I_{CRAC}. Other metabolites in the hexosamine biosynthetic pathway were also included in the pipette solution and their effect on I_{CRAC} determined. Glucosamine

at 10 mM was partially inhibitory, although this may reflects its conversion to glucosamine-6-phosphate soon after establishing the whole-cell configuration and before cytoplasmic ATP was sufficiently diluted. None of the metabolites downstream of glucosamine-6-phosphate, GlcNAc-6-phosphate, GlcNAc-1-phosphate, nor UDP-GlcNAc, inhibited I_{CRAC} (Figure 7).

EXAMPLE 14

Effects of Exogenous Calcium Influx Factor on I_{CRAC}

Cellular extract containing calcium influx factor are able to reduce the latency of activation of I_{CRAC} compared to that seen with 10 μM IP_3, an agent that first releases calcium from intracellular stores, or compared to a cellular extract that does not contain CIF. Activation of I_{CRAC} in patch-clamped cells by this extract is of comparable magnitude to that activated by IP_3, although the rapidity of activation is consistent with a model in which CIF acts in the signaling cascade at a step downstream of the action of IP_3. The current activated by the CIF-containing extract was inwardly rectified with a reversal potential near +60 mV, was less permeable to barium than calcium, and had a 1,000 fold selectivity for calcium over sodium. These properties are consistent with that of I_{CRAC} and the data are consistent with a model of CIF acting at the plasma membrane.

EXAMPLE 15

Relevance Of Data From Diabetic Patients To The Possible Efficacy Of Glucosamine

The data presented demonstrate that peripheral blood mononuclear cells (PBMCs) prepared from diabetic patients show

an impaired calcium signaling pathway. However, whether glucosamine-6-P or increased flux through the hexosamine biosynthetic pathway as being responsible for the observed effects still needs to be investigated.

PBMCs from diabetic patients secrete less NF-AT-mediated cytokines in response to stimuli than do cells from euglycemic control: PBMCs were prepared from adult diabetic outpatients (15.1 ± 1.7 mM blood glucose; significantly elevated glycosylated hemoglobin and from healthy euglycemic volunteers (5.4 ± 0.3 mM blood glucose). Following their isolation from individuals with Type I diabetes or from age- and sex-matched controls, PBMCs were stimulated with phorbol myristate acetate (PMA) and a mitogenic lectin or thapsigargin. The production of four cytokines regulated in part by NF-AT family members as well as IFNγ was assessed 24 hr later (Figure 9). The secretion of IL-2, IL-4, IL-10, and GM-CSF was activated by all stimuli in the control cultures, but stimulated cells from the diabetic individuals produced significantly lower levels of each. In contrast, IFNγ was not effectively stimulated by thapsigargin, and in the cultures stimulated by lectins there were no significant differences between the diabetic and control populations.

PBMCs from diabetic patients have reduced CCE in response to phytohemagglutinin (PHA) relative to cells prepared from euglycemic controls: Because of the dependence of the affected set of cytokines on NF-AT transcription factors, populations of PBMCs from these individuals were loaded with Fura-2 and assessed for $[Ca^{2+}]_i$ responses to PHA. As shown in Figure 10, PHA caused a rapid increase in $[Ca^{2+}]_i$ that reached an average sustained plateau approximately 350 nM greater than

baseline. In contrast, $[Ca^{2+}]_i$ in cells from the diabetic group was only modestly elevated. To determine if the defect associated with diabetes was related to the IP_3-mediated release of Ca^{2+} from intracellular stores, thapsigargin (Tg) was utilized to efficiently deplete these stores and thus induce CCE independent of receptor-mediated signaling. The thapsigargin-induced increase in $[Ca^{2+}]_i$ was also greatly reduced in diabetic patients (Figure 11). A Ca^{2+} removal/Ca^{2+} addback protocol was next utilized in order to separately assess $[Ca^{2+}]_i$ responses due to release of Ca^{2+} from intracellular stores and those due to capacitative influx. Fura-2 loaded PBMCs were suspended in a nominally Ca^{2+}-free buffer. PHA was added, and once $[Ca^{2+}]_i$ returned to baseline extracellular Ca^{2+} was added to 1.25 mM. Ca^{2+} released from intracellular stores was comparable. In control PBMCs the Ca^{2+} addition resulted in an increase in a plateau value of $[Ca^{2+}]_i$ near 600 nM (Figure 12). PBMCs from diabetic individuals again showed only modest increases.

Peripheral blood T lymphocytes from diabetic individuals display minimal I_{CRAC} activation upon passive dialysis: The inhibition of CCE detected utilizing Ca^{2+}-sensitive dyes in cells from diabetic individuals suggested that I_{CRAC}, the plasma membrane current responsible for this influx in lymphocytes (Zweifach et al., 1993), should also be diminished. T lymphocytes were isolated from control and diabetic individuals, and plasma membrane Ca^{2+} currents were examined using whole-cell patch clamping. The patch was achieved with an intra-pipette solution buffered to 20 nM Ca^{2+} with a Ca^{2+} chelator. Under these conditions depletion of ER Ca^{2+} stores leads to spontaneous activation of I_{CRAC} (Parekh et al., 1997). With 10 mM Ca^{2+} present

outside the cell an I_{CRAC} of approximately 3 pA developed over the minutes following the establishment of the patch. I_{CRAC} was seen in T lymphocytes from all control individuals examined and displayed the previously established properties of this current, including a selectively for divalent over monovalent cations, outward rectification with a reversal potential of +30 to +60 mV, and sensitivity to lanthanides (data not shown). In identically prepared T lymphocytes from Type I diabetic individuals, the average I_{CRAC} that developed was less than 1 pA (Figure 13). This deficiency in I_{CRAC} provides a molecular explanation for the absence of CCE seen with Ca^{2+}-sensitive dyes in intact cells and for the absence of the stimulus-induced production of NF-AT dependent cytokines observed in cells from diabetic individuals.

EXAMPLE 16

Glucosamine Lowers the Incidence and Lessens the Severity of Experimental Allergic Encephalomyelitis (EAE)

As a preliminary test of the hypothesis that glucosamine acts as a more general immunosuppressive agent as opposed to a metabolic precursor for enhanced tissue repair, its effects on the development of a model of the human autoimmune disease multiple sclerosis (MS), EAE in Lewis rats was evaluated. In this experiment, groups of five Lewis rats received the indicated concentration of glucosamine in PBS by intraperitoneal injection one hour prior to EAE induction and at daily intervals thereafter. Disease severity was assessed by the following scale: 0=no disease; 1=limp tail; 2=hind limb weakness; 3=paraplegia; and 4=moribund condition or death. As shown in Table 1, glucosamine in a dose-dependent fashion resulted in a 40%

reduction in disease incidence and a 50% reduction in disease severity. These results suggest that *in vivo* effects of glucosamine are not limited to functioning as a biosynthetic precursor involved in tissue repair, and that its central mode of action may well be as an immunosuppressive agent.

TABLE 1

Effects of glucosamine on EAE

Experimental Group (n=5 in each)	Average Day of Disease Onset	Incidence of EAE	Average Severity of EAE
I - 100 mg glucosamine	10.3	3/5	1.2
II - 25 mg glucosamine	11.2	5/5	2.2
III - PBS control	11.4	5/5	2.4

EXAMPLE 17

A Peptide That Binds Calcium-Coordinating Motifs Inhibits I_{CRAC}

Agents that block calcium entry often are designed to act on the channel's pore, whereas compounds that inhibit the activity of molecules responsible for opening the channel also represent a class of potential targets that could be utilized in the design of calcium channel blockers that serve as immunosuppressants. The eight amino acid peptide CALP1 was shown to directly inhibit both calcium entry through non-selective cation channel and I_{CRAC} (Figure 14). This peptide was designed to

bind the E-F hand motif that is responsible for coordinating calcium binding in a variety of proteins. Introduction of CALP1 rapidly inhibited I_{CRAC} and calcium entry via the non-selective cation channel in patch-clamp experiments using Jurkat T cells. These data illustrate a second class of compounds, the peptide CALP1, which is able to effectively inhibit the calcium current activated by CIF.

EXAMPLE 18

Effect of an Inhibitor of CIF Synthesis or Action on Antigen-Specific Proliferation and Th1/Th2 Bias

The effects of glucosamine, an inhibitor of CIF action, on the differentiation of T helper cells to Th1 and Th2 subsets is shown in Figure 15. Peptide 100-116 of the α chain of the nicotinic acetylcholine receptor is a primary autoantigen in an animal model of myasthenia gravis and was utilized to elicit antigen-specific immune responses. The susceptible Lewis rat was injected with the peptide, and nine days later lymph nodes were harvested. The lymphocytes were then stimulated *in vitro* with the peptide with or without selected concentrations of exogenous glucosamine. Three days later the cells were pulsed with (^{3}H)thymidine to examine antigen-dependent proliferation, and in parallel cultures the supernatants were harvested and assessed for cytokine production by ELISA. Glucosamine, an inhibitor of CIF action, caused a marked shift in cytokine production.

In one set of experiments utilizing cultures prepared from each of five injected rats, the presence of 2 mM glucosamine caused no significant change in (^{3}H)thymidine incorporation (Figure 15A) but caused a significant shift toward a Th1 bias, as

assessed by the increased IL-2 and decreased IL-10 production at 72 hr (Figure 15B). These experiments were replicated with five rats and using a range of glucosamine concentrations and incubation times. In cultures from all five rats and at all glucosamine concentrations examined, the production of IL-2 at 18 hours was about 50% above control levels (Figure 16). More striking, however, were the data at 48 and 72 hr. At 48 hr IL-2 production remained elevated with glucosamine, while control cultures began to decrease. At 72 hr in the control, IL-2 had nearly disappeared. In the presence of glucosamine at 1-4 mM the IL-2 levels remained greater than the maximal level seen in the control group. The prolonged presence of IL-2, especially in the face of the ongoing inhibition of IL-4 synthesis, may create a situation in which expression of a Th1 phenotype is unchecked by Th2 responses, and therefore by 72 hrs is remarkably robust. The data provide evidence of a profound effect of glucosamine, an inhibitor of CIF action, on T lymphocyte differentiation pathways.

EXAMPLE 19

The Presence of CIP in Hypertrophied Hearts

The present study also demonstrated that CIF is active in excitable cells, in particular cardiac myocytes. The rationale for examining this tissue has its base in the importance of NF-AT gene transcription as a common feature of cardiac hypertrophy (Molkentin, et al. 1998). It was determined that cultured murine neonatal cardiomyocytes, when treated with angiotensin II, thapsigargin, or endothelin 1, all make CIF while untreated cardiomyocytes do not. In addition, extracts prepared from cardiac tissue taken from dogs in which cardiac hypertrophy due

to chronic volume overload was induced by mitral regurgitation were examined. This treatment results in chronically elevated levels of $[Ca^{2+}]_i$ (Marban, et al. 1987). An increased CIF activity was present in extracts prepared from all dogs with hypertrophied cardiac tissue compared to controls (Figure 17). Because the presence of CIF leads to elevations in $[Ca^{2+}]_i$ that are responsible for NF-AT3-mediated transcription, it follows that inhibitors of CIF will be effective therapeutics in the treatment of cardiac hypertrophy.

Summary

Millimolar concentrations of extracellular glucosamine effectively inhibit capacitative calcium entry. This inhibition is not related to the generation of IP_3, as it was observed when intracellular Ca^{2+} stores were depleted with Tg. The effect was observed in Jurkat and normal primary T cells and BHK-21 cells, as well as in other cell lines examined. The physiological consequences of decreased capacitative calcium entry included inhibited NF-AT nuclear translocation in response to stimuli and a consequent reduction in NF-AT-mediated transcription of cytokines such as IL-2. Both of these deficiencies were reversed when Ca^{2+} was raised by means other than stimulus-induced capacitative calcium entry, e.g. treatment with the Ca^{2+} ionophore A23187.

Several mechanisms could be proposed as being responsible for the inhibitory effect of glucosamine on capacitative calcium entry. In a recent study, Hresko et al. (Hresko et al., 1998) reported that glucosamine treatment of 3T3-L1 adipocytes led to a decrease in total cellular ATP and a resulting inhibition of

insulin-stimulated protein phosphorylation. As a result of their finding, these authors proposed that any biological effect of glucosamine must consider ATP depletion as the cause. However, brief glucosamine treatments under the experimental conditions used here did not significantly alter total cellular ATP levels in Jurkat T cells or in murine macrophage-like J774A.1 cells. The most logical explanation for the lack of ATP depletion by glucosamine is that 5 mM glucose was continually present in all experiments. In contrast, in most of the experiments reported by Hresko et al. (Hresko et al., 1998), the cells were glucose starved for hours prior to glucosamine treatment.

In addition, the inhibition of CCE in J774A.1 cells caused by ATP depletion was reversed by the protein kinase inhibitor staurosporine. However, glucosamine-inhibited CCE in this cell line was not reversed by staurosporine. It is also relevant that after whole cell break-in, when intracellular ATP concentrations are reduced greatly as the cytosol is equilibrated with a large intra-pipette volume that does not contain ATP, I_{CRAC} is activated. Under these conditions, glucosamine-6-phosphate is still a potent inhibitor of I_{CRAC}.

A more likely explanation than ATP depletion is that glucosamine-6-phosphate, an intracellular metabolite common to hexosamine biosynthesis from glucose metabolism and from exogenous glucosamine treatment, inhibits capacitative calcium entry. Using whole-cell patch clamp methods in both Jurkat T cells and RBL-2 cells, it was determined that glucosamine-6-phosphate inhibits I_{CRAC} when present in the intracellular, but not the extracellular, solution. Additionally, glucosamine itself does not inhibit I_{CRAC} unless hexokinase and ATP are present in the

pipette. These data strongly support the idea that glucosamine-6-phosphate is an intracellular inhibitor of I_{CRAC}. It is not known yet whether glucosamine-6-phosphate also inhibits other store-operated Ca^{2+} channels that are present in the plasma membranes of these and other cells. The mechanism of inhibition of I_{CRAC} by glucosamine-6-phosphate could be either through a direct interaction with the plasma membrane channel that is responsible for Ca^{2+} influx, or alternatively, through interaction with a regulator of this channel.

One issue is whether intact cells accumulate glucosamine-6-phosphate to the concentrations required for inhibition of capacitative calcium entry. In the Fura-2 experiments presented here, glucosamine effectively inhibits capacitative calcium entry at extracellular concentrations between 2.5 and 25 mM. Whole-cell patch clamp recordings show that intracellular glucosamine-6-phosphate at concentrations ranging between 100 μM and 10 mM inhibit I_{CRAC}. The experiments with radioactive glucosamine indicate that when extracellular glucosamine was 10 mM, the only glucosamine metabolites present in the cytosol were glucosamine and glucosamine-6-phosphate, and that the latter had an overall cellular concentration of approximately 200-500 μM. When these data are juxtaposed with the patch-clamp recordings and Fura-2 data, it suggests that local concentrations of glucosamine-6-phosphate near the plasma membrane may be significantly greater than those in the rest of the cytoplasm. This could be due to the subcellular localization of hexokinase, the enzyme responsible for the phosphorylation of sugar substrates, at or near the plasma membrane of cells. It has been reported that 40% of hexokinase

activity is localized to the cytoplasmic side of plasma membranes (Daum et al., 1988), and the enzyme has also been shown in macrophages to translocate from the cytoplasm to the plasma membrane following activation (Pedley et al., 1993).

The ability of glucosamine to alter $[Ca^{2+}]_i$-dependent responses including NF-AT-mediated cytokine expression may have important implications to the lymphoid homeostasis and other responses of the immune system. There are four known NF-AT genes that encode the cytoplasmic subunits of the NF-AT complex, NF-ATc, NF-ATp, NF-AT3, and NF-AT4. Each of these can bind to and transactivate multiple target cytokine genes, but their tissue specificity, induction of their own mRNAs upon stimulation and DNA binding affinities are different suggesting that they are functionally distinct (Timmerman et al., 1997). Using genetically engineered mice that lack functional NF-ATp and NF-AT4, Ranger et al. (Ranger et al., 1998) showed that the balance of T helper subpopulations was shifted to that of a Th2 phenotype and that the threshold for activation of cytokine expression was substantially lowered. It is not known whether the threshold for stimulus-induced nuclear translocation of individual NF-AT family members is due to differential sensitivity to $[Ca^{2+}]_i$. It is clear, however, that other cytokine transactivators such as JNK and NF-κB are differentially sensitive to $[Ca^{2+}]_i$ when compared to NF-AT (Dolmetsch et al., 1997).

Increased flux through the hexosamine biosynthesis pathway (Figure 2) and the accumulation of its metabolites, as a result of either hyperglycemia or dietary glucosamine, may at least in part account for the altered immune function observed under these conditions. In a study by Rayfield et al. (1982) a

striking correlation was found between prevalence of infection and mean plasma glucose levels in a diabetic outpatient population. More recently, a retrospective review (Zerr et al., 1997) of patients who underwent cardiac surgery showed up to a fifteen-fold greater risk of deep sternal wound infection with increasing blood glucose in the diabetic population relative to controls. After implementation of a glucose control protocol, there was a 40% drop in infection rates in the diabetic population. The rate of infections remained stable in non-diabetic subjects, suggesting that the decrease was unlikely to be due to other factors. The secretion of selected NF-AT mediated cytokines by peripheral blood mononuclear cells (PBMCs) prepared from diabetic individuals is decreased in response to stimuli compared to those prepared from euglycemic individuals. The decreased secretion of IL-2, IL-4, IL-10, and GM-CSF was attributable to reduced CCE that occurred following stimulation. As this would predict, purified T cells from diabetic individuals were impaired in the activation of the plasma membrane channel responsible for I_{CRAC}. A compromise in $[Ca^{2+}]_i$ regulation could have its effect on infectivity by directly inhibiting monocyte responsiveness or indirectly by creating a cytokine environment in which monocyte function was suboptimal.

It is not just with diabetes that hyperglycemia is an apparent risk factor for increased rates of infection. Patients who have sustained traumatic injuries, particularly burns, and those receiving steroids or total parenteral nutrition (TPN) suffer from increases in the incidence of infections that correlate with hyperglycemia (Black et al., 1990; Pomposelli and Bistrian, 1994). In a report by The Veterans Affairs TPN Cooperative Study Group

of malnourished surgical patients, TPN and the accompanying hyperglycemia led to a greater than two-fold incidence of major infectious complications during the first 30 postoperative days (14.1 vs. 6.4%). Also, as part of the treatment for end-stage renal failure, it is a common practice to dialyze the peritoneum with a solution containing glucose at concentrations exceeding 200 mM. Infection is a major cause of mortality in this dialysis population (Krediet, 1998), and the severity of infection has been suggested to be partially attributed to the excessive glucose in the dialysis solution (Cendoroglo et al., 1998).

Glucosamine sulfate is currently being used as an alternative remedy for osteoarthritis (da Camara and Dowless, 1998). Although the data are limited, the clinical benefit of glucosamine sulfate in several relatively small studies is a reduction in pain that was at least equivalent to that observed in patients treated with ibuprofen (Qiu et al., 1998). Again, the mechanism that underlies the beneficial action of glucosamine in this pathology has not been established. It has been postulated to be due to an increase in the synthesis of extracellular matrix molecules that participate in tissue repair (Setnikar, 1992), although the experimental evidence to support this claim are limited. An equally compelling argument can be made to support the hypothesis that glucosamine-6-phosphate levels are increased in immune cells and lead to inhibited expression of inflammatory cytokines, although measurements of intracellular glucosamine metabolites of cells from arthritic joints have not yet been performed. It is tempting to speculate that glucosamine's beneficial effects in the treatment of osteoarthritis is a result of an altered balance in cytokine expression due to changes in the

transcription factors responding to stimuli as a consequence of lowered $[Ca^{2+}]_i$ and/or a shift in the proportions of T cell subpopulations.

Calcium influx factor (CIF) is likely to be active in cells other than cells of the immune system, such as cardiac myocytes. Therefore, selectivity of CIF action might be a problem and unanticipated side effects might emerge. However, these limitations could be overcome by targeting the drug to immune cells. Alternatively, this might not be an issue since immune cells appear to be more sensitive to CIF's inhibition than are other cell types.

The present invention also demonstrated that glucosamine, an inhibitor of CIP action, had a profound effect on T lymphocyte differentiation pathways and that CIP was present in extracts from hypertrophied hearts. This indicates that inhibitors of CIF action or synthesis could be considered as novel therapeutic agents with respect to cardiac hypertrophy as well as immunosuppression or immunomodulation, due in part to their ability to inhibit NF-AT-mediated signaling.

The following references may have been cited herein:

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and

obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A pharmaceutical compound, wherein said compound inhibits synthesis and/or action of calcium influx factor.

2. The pharmaceutical compound of claim 1, wherein said calcium influx factor is required for capacitative calcium entry and the downstream effects attributable to capacitative calcium entry.

3. The pharmaceutical compound of claim 1, wherein said compound is selected from the group consisting of glucosamine, metabolites of glucosamine, a peptide that binds calcium-coordinating motifs and a compound that inhibits the activity of molecules responsible for opening calcium channel.

4. The pharmaceutical compound of claim 3, wherein said metabolite of glucosamine is glucosamine-6-phosphate.

5. The pharmaceutical compound of claim 3, wherein said peptide is CALP1 having the amino acid sequence shown in SEQ ID No. 1.

6. The pharmaceutical compound of claim 3, wherein inhibition by said compound is concentration-dependent.

7. A method of suppressing immune responses in an individual in need of such treatment, comprising the step of:

administering the pharmaceutical compound of claim 1 to said individual.

8. The method of claim 7, wherein said individual suffers from the condition selected from the group consisting of transplant rejection, autoimmune diseases, inflammation associated diseases and a condition in which immunosuppression is therapeutic.

9. The method of claim 8, wherein said autoimmune disease is selected from the group consisting of arthritis, asthma, multiple sclerosis and diabetes.

10. The method of claim 8, wherein said inflammation associated disease is osteoarthritis.

11. The method of claim 7, wherein said pharmaceutical compound inhibits Nuclear Factor of Activated T cells (NF-AT)-mediated gene expression and other calcium-sensitive transcription pathways.

12. The method of claim 11, wherein said gene is selected from the group consisting of Interleukin-2, Interleukin-4, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Fas ligand and CD-40.

13. A method of treating an individual having an impaired immune response condition, comprising the step of:

increasing intracellular free calcium concentration ($[Ca^{2+}]_i$) in selected cells of said individual, wherein said cells are associated with said impaired immune response condition.

14. The method of claim 13, wherein said impaired immune response condition is caused by hyperglycemia or dietary glucosamine.

15. The method of claim 14, wherein said hyperglycemia is associated with the condition selected from the group consisting of diabetes, traumatic injuries, steroid treatment, total parenteral nutrition treatment and end-stage renal failure treatment which utilizes excessive glucose in the dialysis solution.

16. A method of treating an individual having an impaired immune response condition, comprising the step of:

blocking hexosamine biosynthesis pathway in selected cells of said individual, wherein said cells are associated with said impaired immune response condition.

17. The method of claim 16, wherein said impaired immune response condition is caused by hyperglycemia or dietary glucosamine.

18. The method of claim 17, wherein said hyperglycemia is associated with the condition selected from the group consisting of diabetes, traumatic injuries, steroid treatment, total parenteral nutrition treatment and end-stage renal failure treatment which utilizes excessive glucose in the dialysis solution.

19. A method of modulating Th1/Th2 balance or altering T cell apoptosis in an individual in need of such treatment, comprising the step of:

administering the pharmaceutical compound of claim 1 to said individual.

20. A method of treating an individual having or in danger of developing cardiac hypertrophy, comprising the step of:

administering the pharmaceutical compound of claim 1 to said individual.

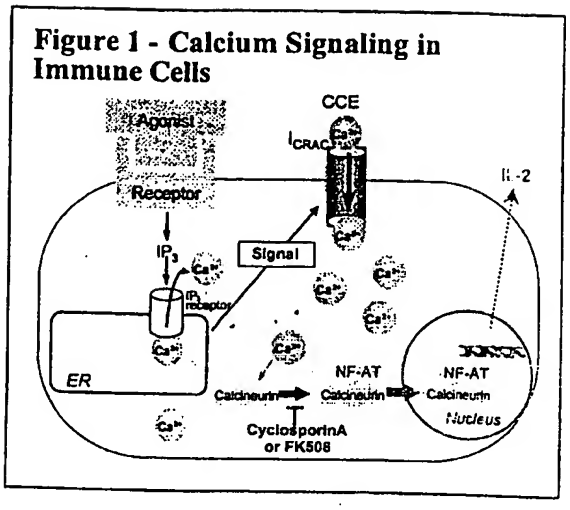


Figure 1 - Calcium Signaling in Immune Cells
CCE
Agonist
Receptor
I_{CRAC}
IL-2
IP_3
Signal
IP_3 receptor
ER
NF-AT
Calcineurin
Calcineurin
NF-AT
Calcineurin
Nucleus
CyclosporinA or FK506

Figure 1

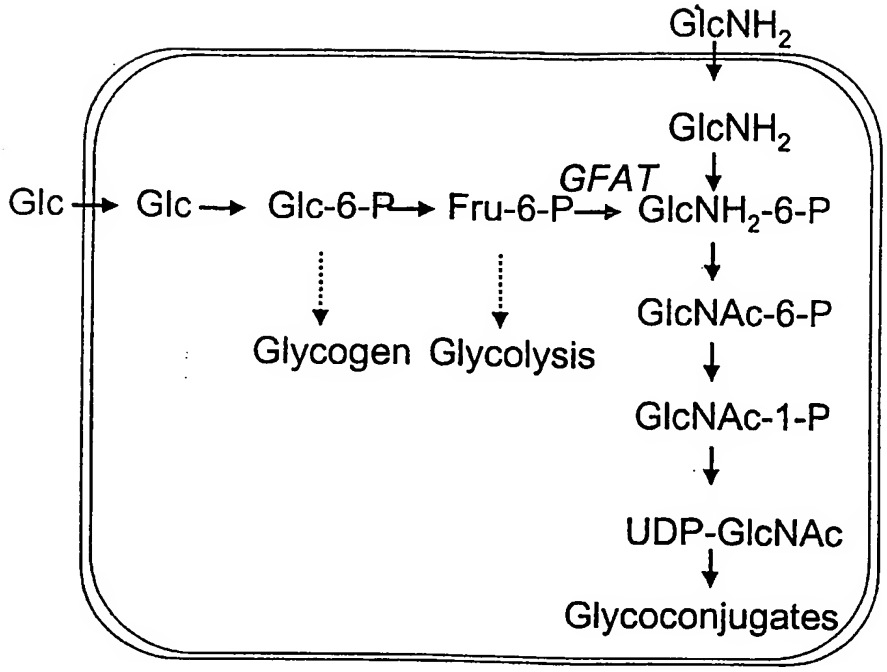


Figure 2

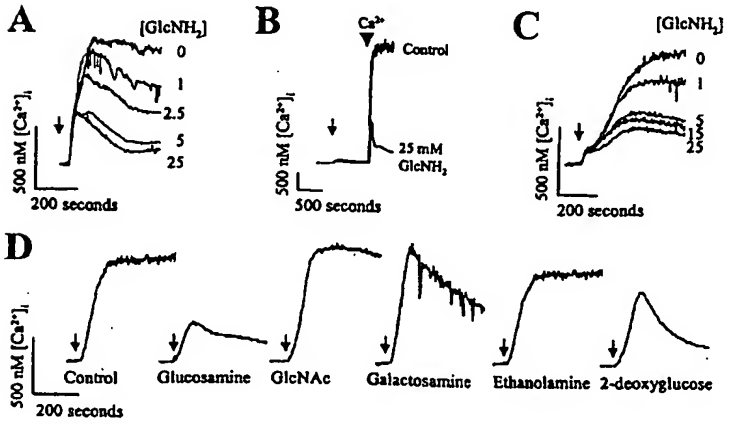


Figure 3

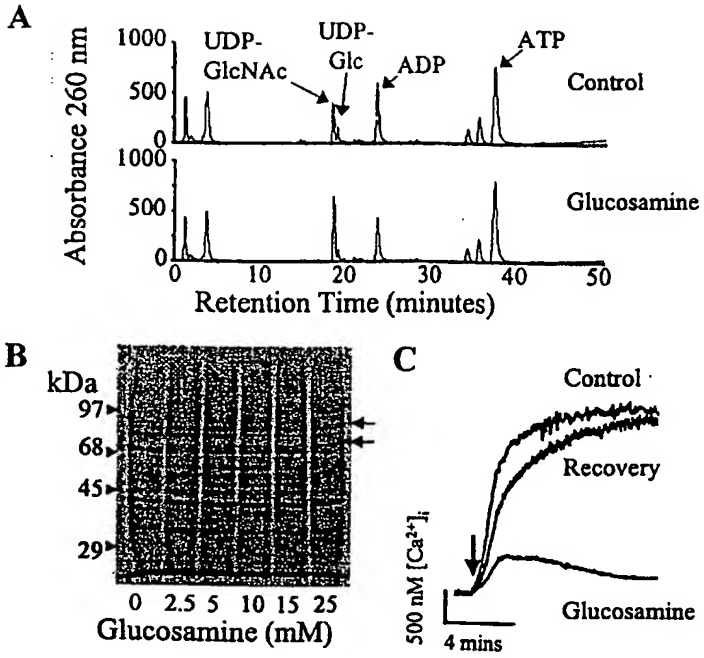


Figure 4

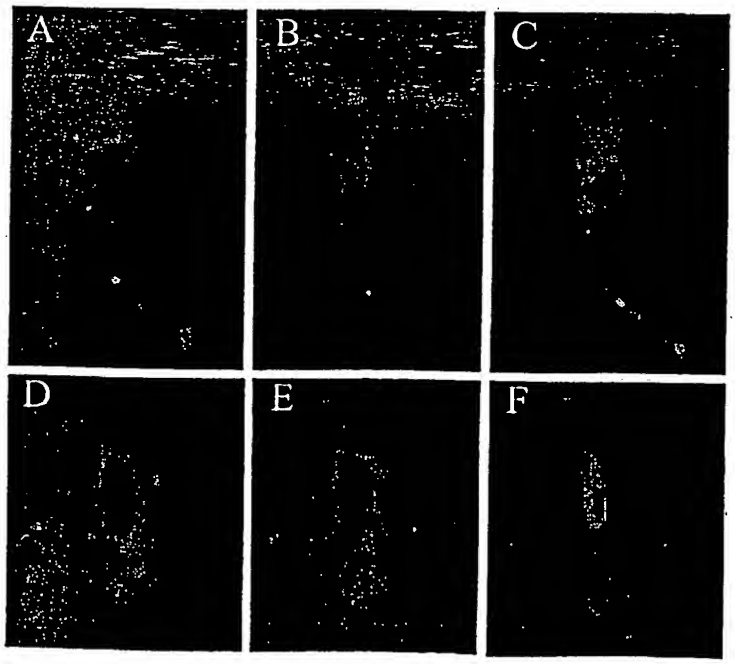


Figure 5

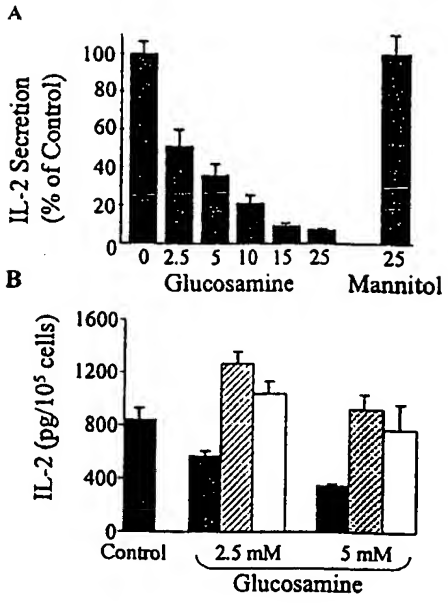

A
IL-2 Secretion (% of Control)
100
80
60
40
20
0
0 2.5 5 10 15 25
25
Glucosamine
Mannitol
B
IL-2 (pg/10^5 cells)
1600
1200
800
400
0
Control
2.5 mM
5 mM
Glucosamine

Figure 6

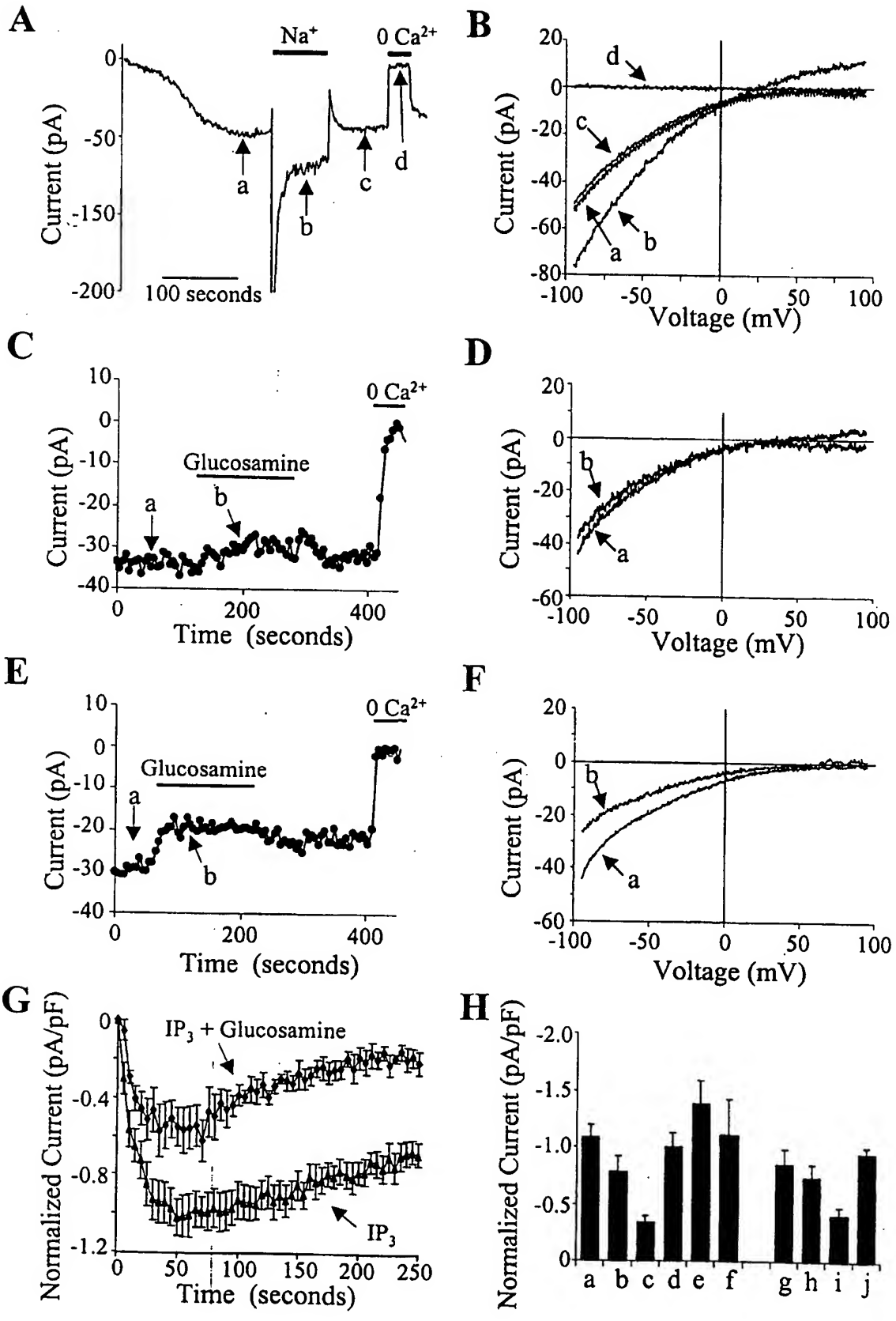

A
Na+
0 Ca2+
Current (pA)
100 seconds
a
b
c
d
B
Current (pA)
Voltage (mV)
C
0 Ca2+
Glucosamine
Current (pA)
Time (seconds)
D
Current (pA)
Voltage (mV)
E
0 Ca2+
Glucosamine
Current (pA)
Time (seconds)
F
Current (pA)
Voltage (mV)
G
IP3 + Glucosamine
IP3
Normalized Current (pA/pF)
Time (seconds)
H
Normalized Current (pA/pF)
a b c d e f g h i j

Figure 7

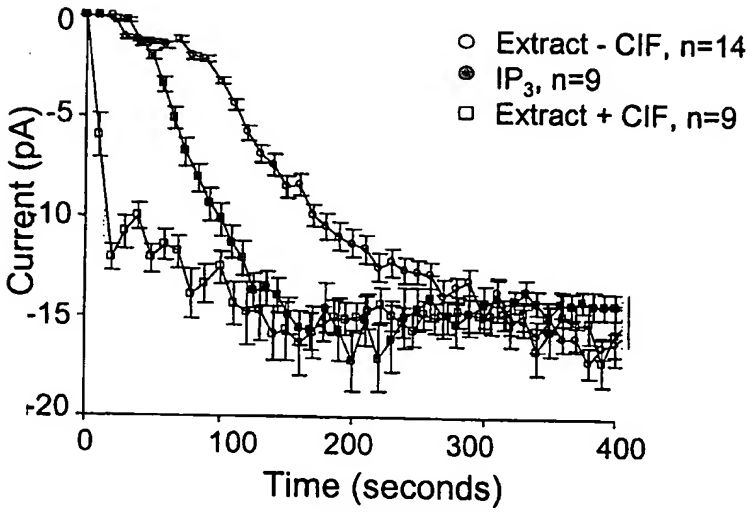

0
-5
-10
-15
-20
Current (pA)
0
100
200
300
400
Time (seconds)
Extract - CIF, n=14
IP3, n=9
Extract + CIF, n=9

Figure 8

Figure 9 - PBMCs from Diabetic Individuals Secrete Lower Levels of NF-AT Mediated Cytokines Following Activation Than Do Those from Controls

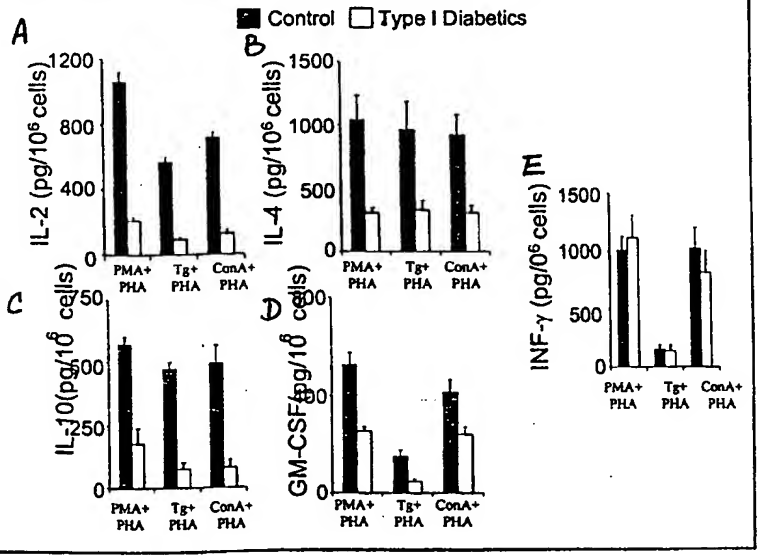


Figure 9

Figure 10 – CCE Elicited by PHA is Decreased in PBMCs Prepared from Diabetic Individuals Compared to Euglycemic Controls

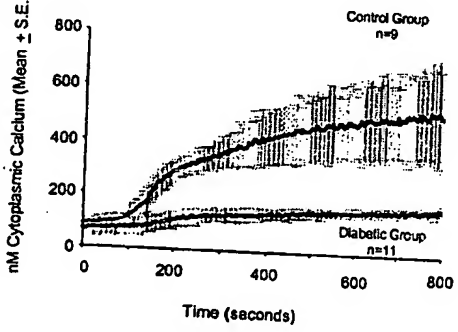


Figure 10

Figure 1 – CCE Elicited by Tg is Decreased in PBMCs Prepared from Diabetic Individuals Compared to Euglycemic Controls

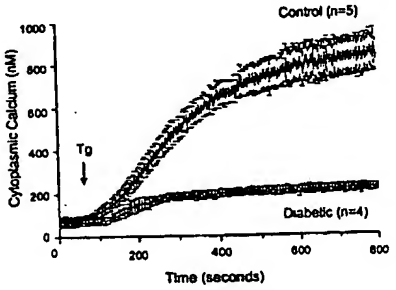


Figure 11

Figure 12 – CCE Elicited by PHA is Decreased in PBMCs Prepared from Diabetic Individuals Compared to Euglycemic Controls

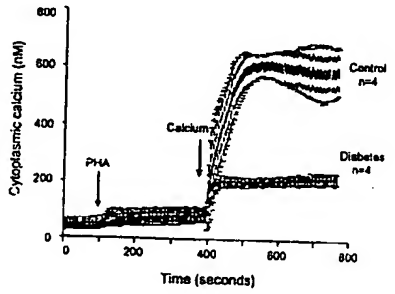


Figure 12

Figure 13 – T Cells from Diabetic Patients Have a Markedly Reduced I_{CRAC}

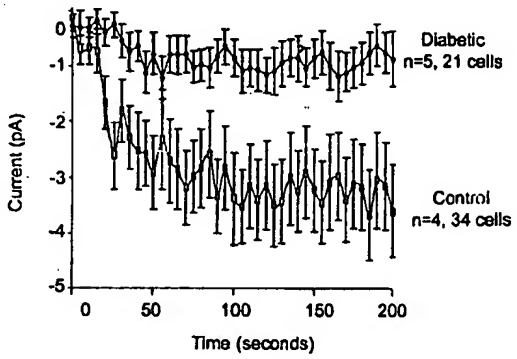


Figure 13

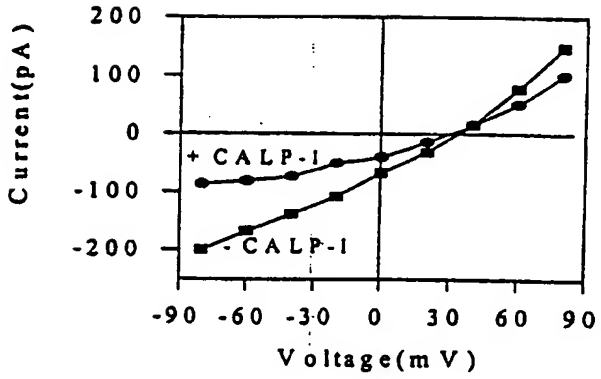


Figure 14A

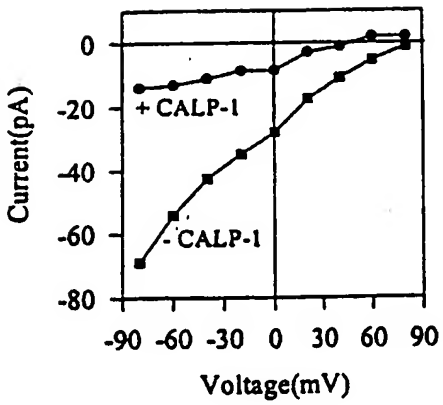


Figure 14B

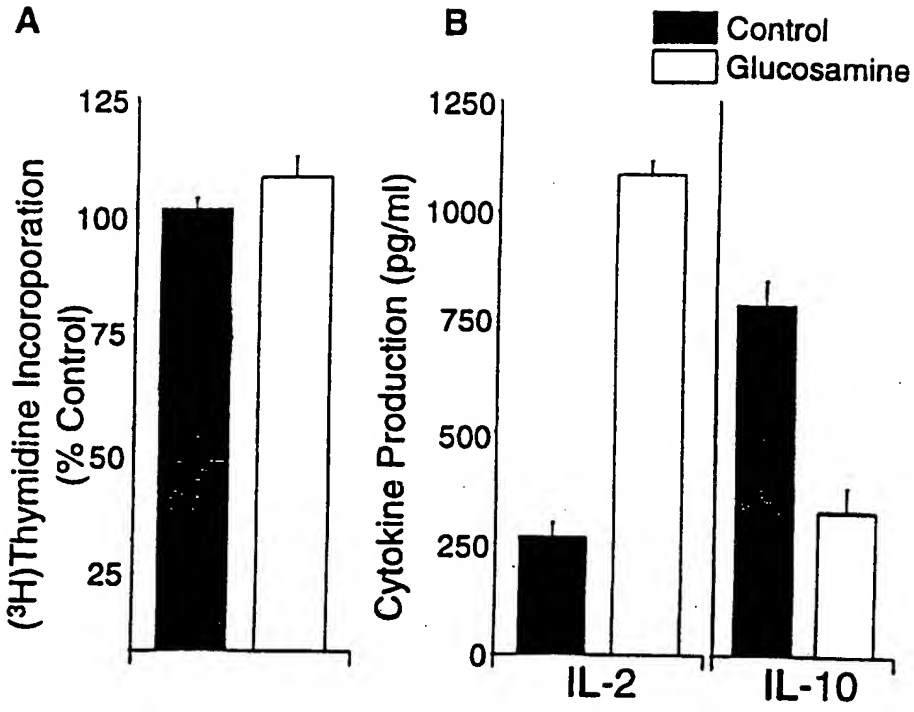

A
B
Control
Glucosamine
125
100
75
50
25
(³H)Thymidine Incoroporation (% Control)
1250
1000
750
500
250
0
Cytokine Production (pg/ml)
IL-2
IL-10

Figure 15

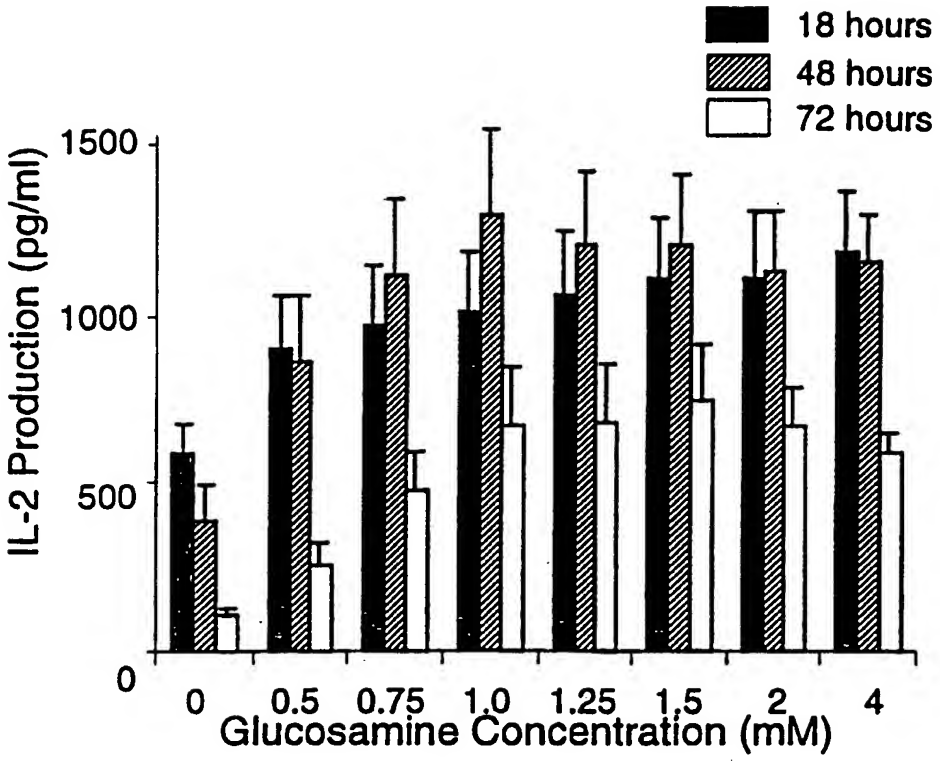
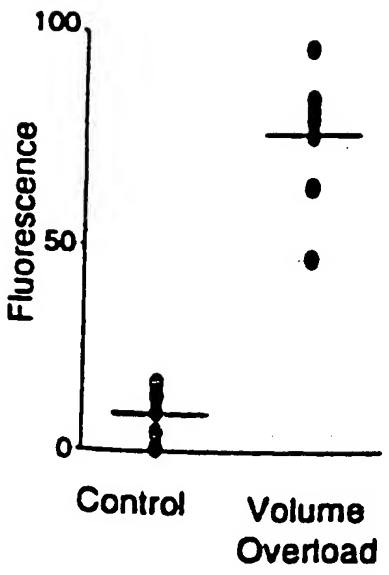

18 hours
48 hours
72 hours
IL-2 Production (pg/ml)
1500
1000
500
0
0
0.5
0.75
1.0
1.25
1.5
2
4
Glucosamine Concentration (mM)

Figure 16



Volume Overload
Cardiac Hypertrophy

- High levels of Ang II and/or endothelin-1
- High levels of $[Ca^{2+}]_i$
- NF AT-dependent gene transcription may be a common feature of the hypertrophic response

Figure 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/06166

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 31/70, 38/08; C07K 7/06
US CL : 514/16, 62; 530/328; 536/55.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/16, 62; 530/328; 536/55.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, CAPLUS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 3,683,076 A (ROVATI) 08 August 1972, see entire document.	1-18
X	US 4,590,067 A (MEISNER) 20 May 1986, see entire document.	1-6
X	US 5,843,919 A (BURGER) 01 December 1998, see entire document.	1-20
X --- Y	DE 4 237 129 A1 (RADULESCU et al.) 05 May 1994, see entire document.	1-6 --- 7-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 JULY 2000	Date of mailing of the international search report 31 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANISH GUPTA Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)★